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Disinfection Resistance of 'Legionella pneumophila' and 'Escherichia coli' Grown in Continuous and Batch Culture

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Disinfection Resistance of *Legionella pneumophila* and *Escherichia coli* Grown in Continuous and Batch Culture

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16. ABSTRACT

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ABSTRACT

Legionella pneumophila and *Escherichia coli* were grown in continuous culture to simulate nutrient-limited, sub-optimal growth conditions that are often experienced in aquatic environments. Washed cultures were treated with chlorine dioxide (ClO_2) or chlorine at a dose equal to 0.75 mg/l. Results showed that the chemostat grown populations of both species were more resistant to inactivation than were their batch-culture grown counterparts. ClO_2 was shown to be superior to chlorine on a mass-dose basis in inactivating *L. pneumophila*. The adequacy of *E. coli*, a major constituent of the coliform group, as an indicator of *Legionella* inactivation depended on the antecedent growth conditions: the inactivation behavior of the two organisms grown in continuous culture was similar, whereas *L. pneumophila* was more resistant than *E. coli* when both were grown in batch culture.

INTRODUCTION

Several Legionella species isolated from a diverse set of aquatic environments[1-8] have been reported to exhibit differing sensitivities to disinfectants.[9,10] Laboratory studies have shown that batch-grown Legionella pneumophila is readily inactivated by chlorine as hypochlorite ion.[11] However, we demonstrated previously that batch-grown cultures of bacteria are more sensitive to disinfectants, and therefore not representative of their naturally-occurring counterparts.[12-14] Nor have any studies been reported that evaluate the efficacy of chlorine dioxide (ClO_2) against Legionella.

The objectives of this study were threefold. First, to assess the sensitivity to chlorine dioxide of L. pneumophila, grown under nutrient limitation in a chemostat. Second, to compare the sensitivity of L. pneumophila and Escherichia coli, a predominant constituent of the widely-accepted coliform indicator group, and third, to contrast the efficacy against L. pneumophila of chlorine dioxide with that of chlorine.

MATERIALS AND METHODS

Cultures of L. pneumophila and E. coli were grown in batch and continuous culture as described previously.[13,15,16] Simulation of nutrient-limited growth at sub-maximal rates was accomplished in a chemostat. Variables were growth temperature and growth rate, while substrate concentration, pH, dissolved oxygen, and agitation rate were held constant.

A special disinfection reactor was built to contain the aerosol-transmitted Legionella (Fig. 1). The vessel was designed to be filled, inoculated, dosed, drained and rinsed aseptically; and to be sampled rapidly and under negative pressure into a biohazard cabinet. Suspensions of cultures at a concentration of ca. 10^6 CFU/ml were dosed with ClO_2 or chlorine to achieve an initial concentration equal to 0.75 mg/l as ClO_2. Survivors were enumerated on CYE agar[11] (L. pneumophila) or m-Endo agar[12] (E. coli). Each set of growth conditions was evaluated in triplicate experiments.[12-16]

A biphasic survivor curve with rapid, initial inactivation followed by a constant plateau was consistently observed. The plateau represents a resistant surviving subpopulation, the size of which is determined by the growth environment. This phenomenon is discussed in detail elsewhere.[12,16,17] The ratio of the size of the surviving fraction at 15 min. (i.e. log N_{15}/N_0) was the basis for comparing the effects of the different environmental variables.

RESULTS

Effect of Growth Rate and Growth Temperature on Sensitivity of L. pneumophila

The results of experiments to assess the effects of growth rate and growth temperature on the sensitivity of L. pneumophila to chlorine dioxide are shown in Table 1. Approximately a five-fold or greater difference in dilution rate was required before a difference in sensitivity was observed at a growth temperature

equal to 37°C. For example, there was a significant difference between the survival ratios at D = 0.03 h^{-1} and D = 0.15 h^{-1}. However, at a growth temperature equal to 44°C, a significant difference in survival ratios was observed over a two-fold difference in growth rate, D = 0.03 to D = 0.06 h^{-1}. Otherwise smaller changes in D do not reveal a substantial difference in sensitivity at either growth temperature.

The basis for comparing the effects of growth conditions on sensitivity was the t-test using alpha = 0.05 with 16 degrees of freedom[18] (Table 2). There was no consistent relationship between growth temperature and sensitivity, although only two temperatures, 37 and 44°C, were studied. Statistically significant differences in sensitivity were found for differences in growth rate (D) of 0.03 vs. 0.06 and 0.06 vs. 0.20 at a growth temperature equal to 44°C. These particular comparisons were chosen to demonstrate that large differences in growth rate are typically required to achieve significant differences in sensitivity.

Comparison of L. pneumophila with E. coli

The rates of initial inactivation and size of the recalcitrant fraction (i.e., log N_{15}/N_0) of L. pneumophila and of E. coli are compared in Figures 2 and 3. A large difference in sensitivity between the two species is revealed when either is grown in batch culture (Fig. 2). The difference is observed in the initial rate of inactivation as well as in the size of the recalcitrant fraction. When chemostat-grown cultures were used,

however, there was a relationship between dilution rate and the size of the recalcitrant fraction that held for both species (Fig. 3). In the experiments chosen for comparison, similar complex media were utilized. L. pneumophila was grown in yeast extract and E. coli was grown in nutrient broth, at the same growth temperature, 37°C. Within the range of dilution rates studied, E. coli and L. pneumophila do not exhibit significantly different sensitivity to ClO_2.

Comparison of Chlorine Dioxide and Chlorine

One experiment was conducted to compare the efficacy of treatments with ClO_2 or chlorine. The results indicate that ClO_2 was more effective than chlorine (Fig. 4) in the inactivation of batch-grown L. pneumophila. Experiments were not conducted with chemostat-grown cultures.

DISCUSSION

Effect of Growth Rate and Growth Temperature on Sensitivity of L. pneumophila

The same trend that had been observed for E. coli grown in a complex medium in the chemostat[12] also was observed for L. pneumophila: namely, increasing growth rate yielded a more sensitive population as characterized by the decreased size of the resistant fraction. The effect of varying the growth rate was less pronounced for L. pneumophila than for E. coli. This may be explained in two different ways: (1) the incremental changes in the growth rate did not affect the physiological "target" for inactivation, or (2) the precision of the

experiments was such that more subtle changes in sensitivity could not be distinguished. The most notable difference in sensitivity is found at the two extremes of dilution rates ($D = 0.03$ and $D = 0.30\ h^{-1}$) studied. If it is assumed that these two conditions more closely approximate the fast growth rate obtained at log-phase in batch culture ($D = 0.30\ h^{-1}$) and the sub-maximal rates achieved in natural systems ($D = 0.03\ h^{-1}$), then the results support the major thesis of the study: conditions more closely resembling nutrient-limited, sub-optimal growth produce bacterial populations that are more resistant to chemical disinfection.

Based on previous experiments with *E. coli*[12], a relationship between growth temperature and sensitivity for *L. pneumophila* was plausable, although none was observed. This may reflect the capability of the organism to grow over a wide range of temperatures with an optimum within the range bracketed by the temperatures that were studied (37 to 44°C). It is reasonable to expect that an organism with a broad range of growth temperatures would not experience physiological changes as a result of a small change in temperature sufficient to effect sensitivity.

Attempts to grow the cultures in the laboratory at higher (50°C) and lower (25°C) temperatures, which were desirable from the perspective of modeling environmental conditions, were of limited success. Growth of a steady-state culture at 25°C was not attained at $D \leq 0.03\ h^{-1}$ (i.e., washout occurred). The culture remained viable at a temperature of 50°C, but a steady

state was not attained. It was determined from the washout rate[19] that the U_{max} at 50°C was approximately equal to D = 0.02 h^{-1}. It has been observed that changes in growth temperature affect the lipid structure and outer membrane permeability[20]. Further studies could verify whether changes in a wider range of growth temperatures affects the sensitivity to chemical disinfectants. L. pneumophila would be an ideal organism, owing to its ability to grow over a wide temperature range in nature.[3]

Comparison of L. pneumophila with E. coli

Similarities in sensitivity to ClO_2 exist between L. pneumophila and E. coli regarding the effect of growth temperature and growth rate in complex media. Both organisms exhibited a general trend towards increasing sensitivity as growth rate, and to a lesser extent, growth temperature, were increased. It is reasonable that both organisms should behave similarly since both are gram-negative organisms possessing similar outer membrane structures. The results of other experiments conducted in this laboratory using Yersinia enterocolitica and Klebsiella pneumoniae support the results obtained for E. coli and L. pneumophila in that growth temperature and growth rate affect sensitivity to ClO_2.[21] We postulate that alterations of the growth environment will affect the sensitivity to chemical disinfectants of other gram-negative, and possibly gram positive, bacteria in an analogous manner.

The sizes of the recalcitrant fractions of L. pneumophila and of E. coli were compared to test the validity of using the

coliform group, of which E. coli is the predominant species, as an indicator of Legionella inactivation. A comparison between the two species (Fig. 2), each grown in batch culture, indicates that L. pneumophila was more resistant to ClO_2 with respect to initial rate and extent of inactivation. The results of this conventional batch-culture procedure lead one to conclude that E. coli is not a conservative indicator of the inactivation of L. pneumophila. However, a comparison between chemostat grown populations (Figure 3) leads to a different conclusion. The apparent linear relationship of the data strongly suggests that: 1) the two species behave similarly over a range of sub-maximum growth rates, and 2) a positive correlation exists between increasing sensitivity to ClO_2 and increasing dilution rate. It can be inferred from these data that E. coli and L. pneumophila exhibit similar sensitivity to ClO_2 under nutrient-limited conditions likely to be found in nature. Therefore, E. coli should be an adequate indicator for the effectiveness of L. pneumophila inactivation by ClO_2. Clearly, further testing would be required, along with in situ verification, to extrapolate these conclusions to real situations.

The fact that such different conclusions can be drawn from either the use of batch grown or chemostat grown cultures is supported by comparing the results of Kuchta, et al[22] and this study. In the former study[22] it is shown that batch grown L. pneumophila was significantly more resistant than E. coli to chlorine, whereas in this study the two species responded

similarly to exposure to ClO_2 following growth in the chemostat.

Comparison of Chlorine Dioxide and Chlorine

The results of these experiments indicate that ClO_2 was superior to chlorine when compared at equal mass-dose. Similar results have been observed by other investigators using other species.[23-28] Since the molecular weights are nearly identical, a comparison on an equimolar basis would yield essentially the same results. While the comparison on a mass-dose or molar basis is practical from an economic perspective, one must consider other factors before concluding whether one disinfectant or the other is superior. If one were to compare the disinfectants on a residual basis instead of a dose basis, the composition of the aqueous solution would be a critical factor. For example, water containing a high concentration of ammonia nitrogen would produce predominantly monochloramine, an inferior bactericide, when treated with chlorine, whereas the chlorine dioxide would be largely unaffected by the presence of ammonia. In that case, comparison on a total residual basis would yield drastically different information than a comparison on a dose basis owing to significant extraneous side-reactions with one disinfectant.

CONCLUSIONS

Chlorine dioxide was effective in inactivating L. pneumophila, using as a criterion the size of the resistant fraction, when compared with chlorine on an equal dose basis. On this basis chlorine dioxide can be considered to be a superior

alternative to chlorine for the control of L. pneumophila in water treatment.

The validity of E. coli as an indicator for the control of L. pneumophila was not supported by experiments using batch-grown cultures. The opposite conclusion could be drawn from the results of the chemostat studies. If the chemostat, as used in this study, is accepted as a laboratory model of natural, aquatic environments, then E. coli would be an acceptable indicator organism. The results, of course, represent only a limited number of laboratory experiments and would need to be verified by further experiments.

The results cast a new light on the customary methods used to evaluate microbial contaminants of concern to public health. The use of the chemostat is an innovative methodology for the evaluation of antimicrobial agents in the laboratory, and may be preferable to the use of conventional batch culture techniques. The simulation in the laboratory of the environmental system of concern can provide populations that more closely approximate the resistance to treatment that can be expected in a field evaluation.

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gratefully acknowledged. This paper has not been subjected to the Agency's required administrative and peer review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The results were presented, in part, at the Second International Symposium on Legionella, Atlanta, Georgia, June 19-23, 1983.

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TABLE 1

Effect of Growth Temperature and Growth Rate on Sensitivity of *L. pneumophila* to 0.75 mg/l ClO_2.

Dilution Rate $\bar{D}$ (h^{-1})	Growth Temp (C°)	Surviving Fraction ($Log(N_t/N_o)$ at t = 15 min)
0.03	44	-2.99 ± 0.46
0.06	44	-4.29 ± 0.37
	37	-4.13 ± 0.36
0.15	44	-4.33 ± 0.32
	37	-5.06 ± 0.40
0.20	44	-4.86 ± 0.49
	37	-4.75 ± 0.45
0.30	37	-5.09 ± 0.30

TABLE 2

Comparison of Selected Values from Table 1 Using t-Test[18] to Determine Any Differences in Sensitivity Resulting from Different Growth Conditions.

Conditions Compared (Temp. or D)	$\bar{Y}_2$	S_2^2	$\bar{Y}_1$	S_1^2	t^*	$t_{[0.05,16]}$	Means Significantly Different
37° vs 44° at D = 0.06	4.13	0.13	4.29	0.14	0.92	2.12	no
37° vs 44° at D = 0.15	4.33	0.10	5.06	0.16	5.72	2.12	yes
0.03 vs 0.06 at T = 44°	2.99	0.21	4.29	0.14	8.79	2.12	yes
0.06 vs 0.20 at T = 44°	4.29	0.14	4.86	0.24	2.77	2.21	yes

$$^*t = \frac{(\bar{Y}_1 - \bar{Y}_2) - (u_1 - u_2)}{\sqrt{\frac{1}{n}(S_1^2 + S_2^2)}};$$

$\bar{Y}_1$ and $\bar{Y}_2$ are observed population means; S_2 and S_1 are observed population standard derivations; u_1 and u_2 are the population means, and n_1 and n_2 are numbers of observations.

where $u_1 = u_2$ and $df = n_1 + n_2 - 2 = 16$

FIGURE CAPTION SHEET

Figure 1. Schematic of one-liter disinfection reactor for *Legionella pneumophila*.

Figure 2. Comparison of inactivation of batch-grown *E. Coli* (o) and *L. pneumophila* (●). Cultures were grown at 37°C and harvested at late log phase. Chlorine dioxide dose was 0.75 mg/l.

Figure 3. Effect of dilution rate on sensitivity to a dose of 0.75 mg/l ClO_2. *L. pheumophila* (●); *E. Coli* (o).

Figure 4. Comparison of chlorine dioxide and chlorine against batch-grown *L. pneumophila*. Dose was 0.75 mg/l as ClO_2 (●) or Cl_2 (o).

Figure 1

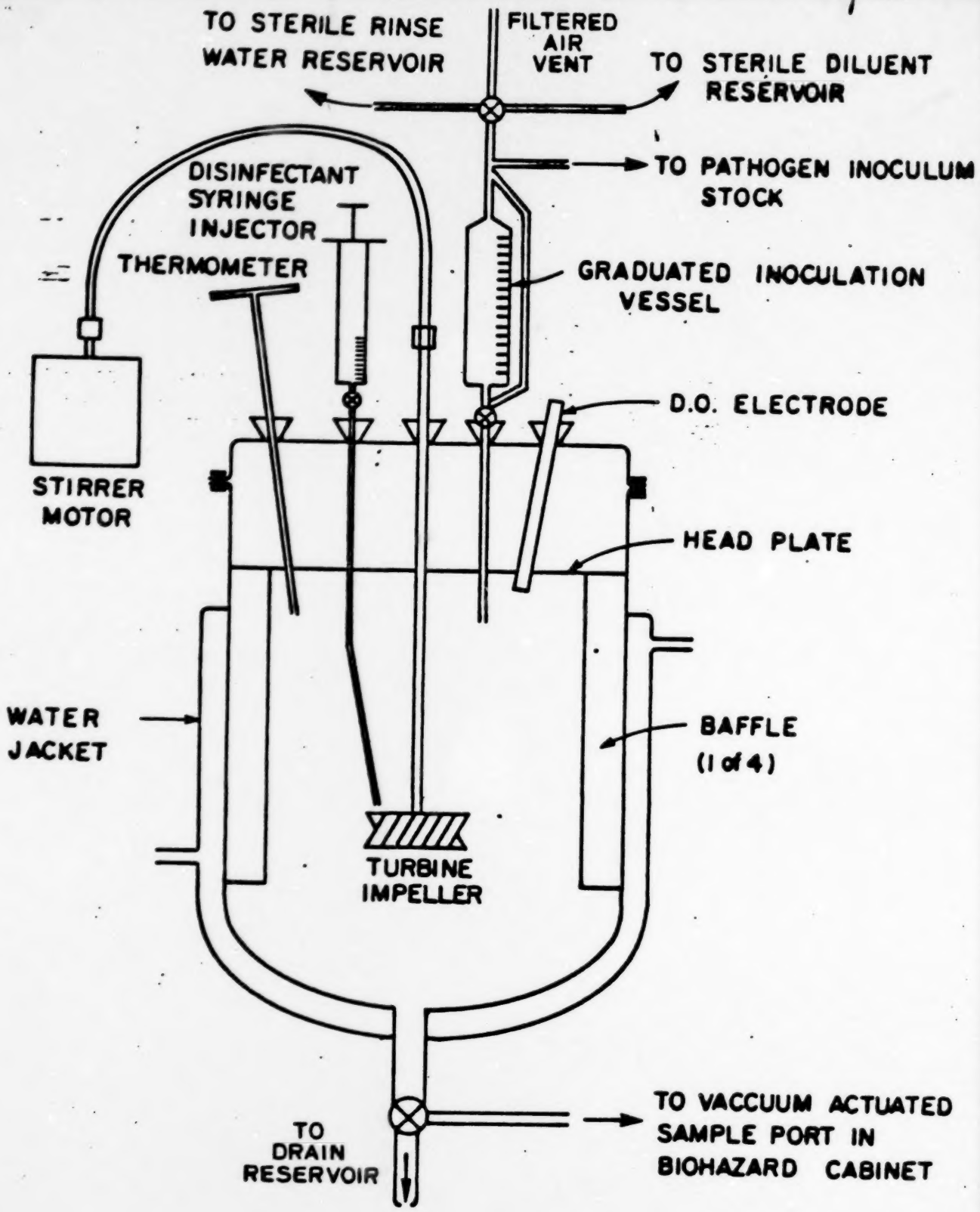
TO STERILE RINSE WATER RESERVOIR
FILTERED AIR VENT
TO STERILE DILUENT RESERVOIR
TO PATHOGEN INOCULUM STOCK
DISINFECTANT SYRINGE INJECTOR
THERMOMETER
GRADUATED INOCULATION VESSEL
D.O. ELECTRODE
STIRRER MOTOR
HEAD PLATE
WATER JACKET
BAFFLE (1 of 4)
TURBINE IMPELLER
TO VACCUUM ACTUATED SAMPLE PORT IN BIOHAZARD CABINET
TO DRAIN RESERVOIR

Figure 2

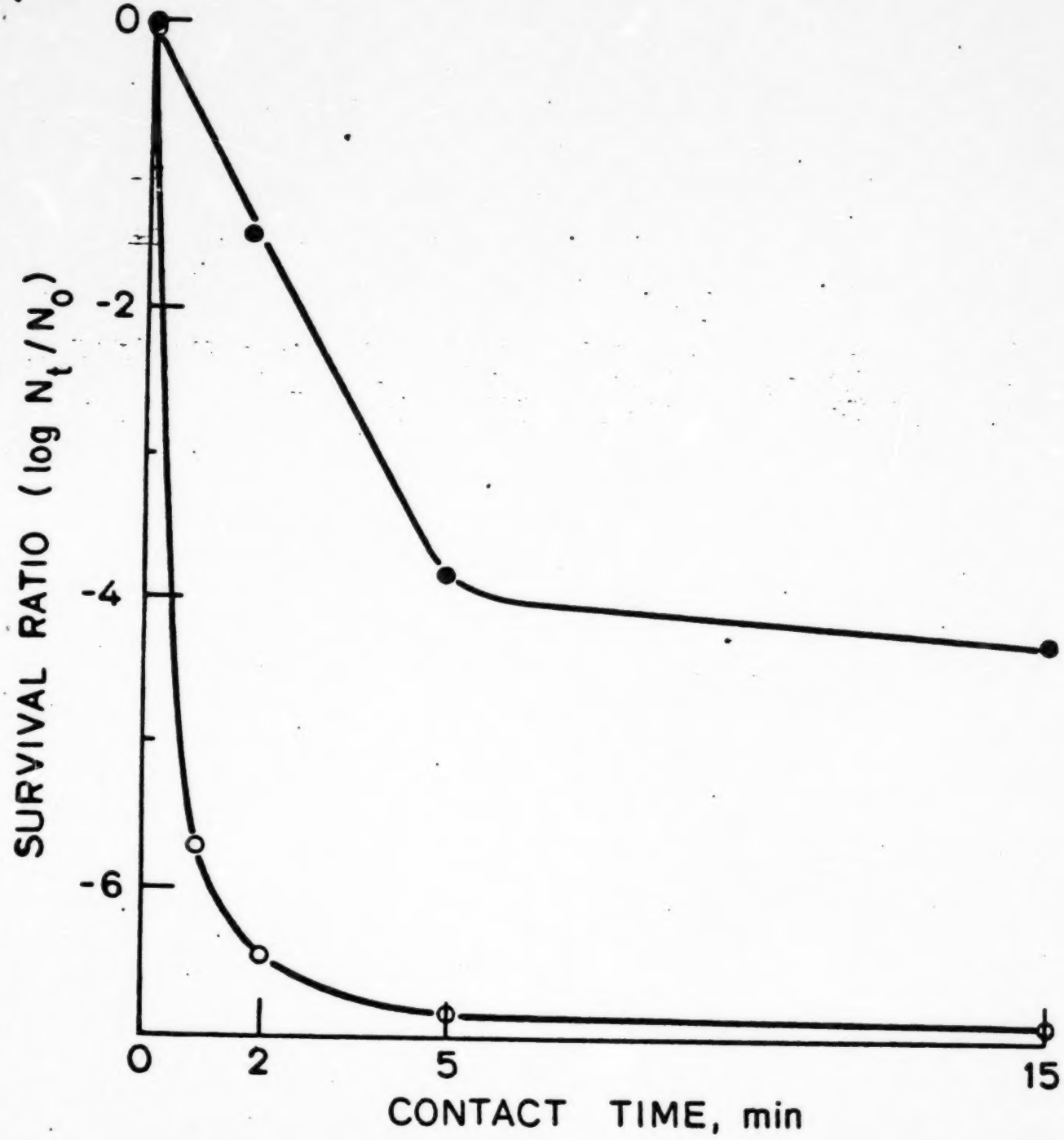

Figure 3

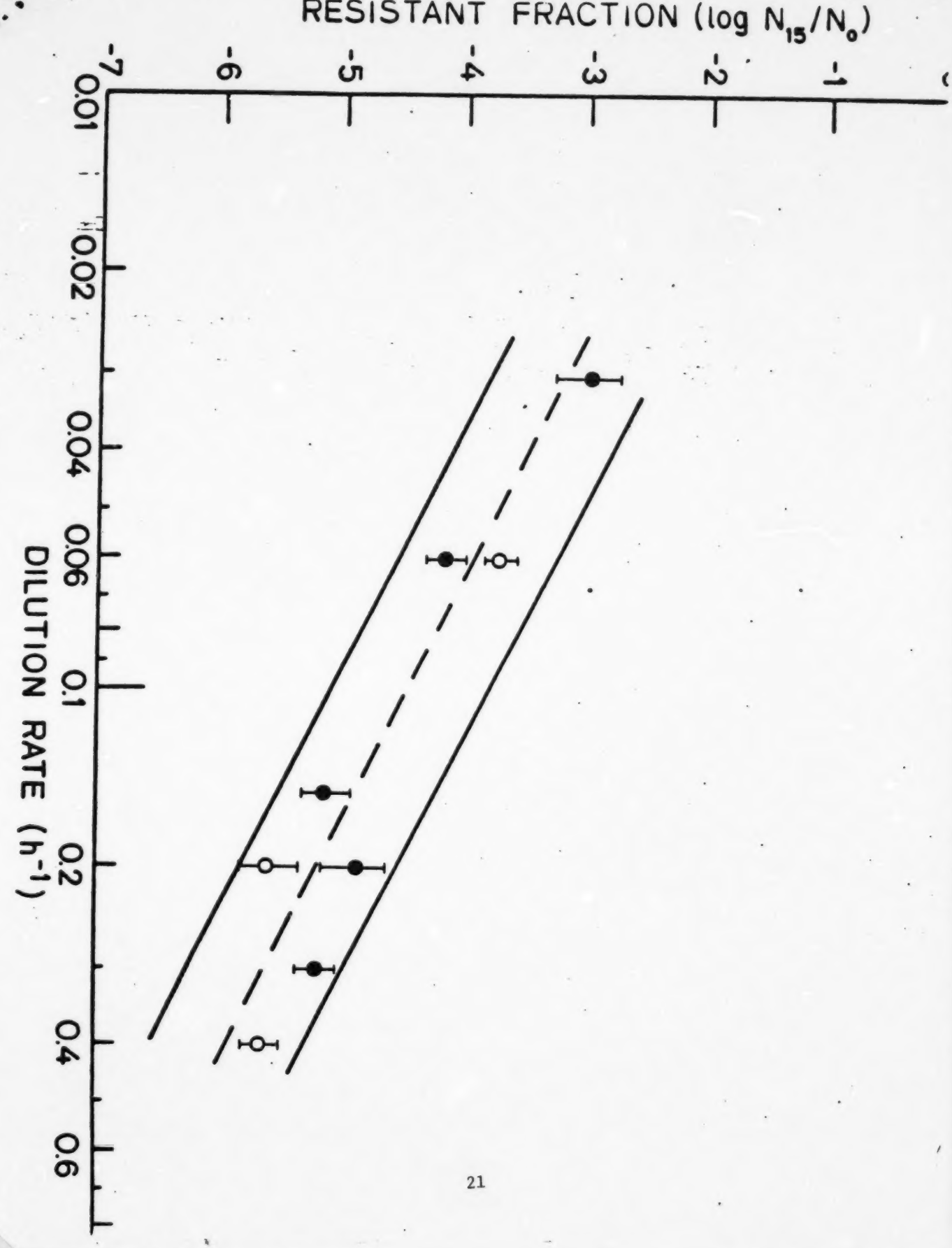
RESISTANT FRACTION (log N_{15}/N_o)
-7
-6
-5
-4
-3
-2
-1
0.01
0.02
0.04
0.06
0.1
0.2
0.4
0.6
DILUTION RATE (h^{-1})

Fig. 4

Figure 4